Acta Veterinaria Hungarica 51 (2), pp. 197–208 (2003)

GROWTH HORMONE AND INSULIN-LIKE GROWTH FACTOR-I ACTION ON PROGESTERONE SECRETION BY PORCINE CORPORA LUTEA ISOLATED AT VARIOUS PERIODS OF THE LUTEAL PHASE

Anna PTAK[1], Ewa L. GREGORASZCZUK[1*] and J. RZĄSA[2]

[1]Laboratory of Physiology and Toxicology of Reproduction, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Cracow, Poland; [2]Department of Animal Physiology, Academy of Agriculture, Cracow, Poland

(Received May 22, 2002; accepted December 10, 2002)

This study was conducted to investigate the interactions between growth hormone (GH) and insulin-like growth factor-I (IGF-I) on progesterone (P4) secretion by porcine luteal cells cultured *in vitro*. Cells isolated from corpora lutea (CL) collected at three different periods of the luteal phase (CL1 – early luteal phase; CL2 – middle luteal phase and CL3 – late luteal phase) were incubated with different doses of GH (10, 100 or 200 ng/ml). After 48 h cultures were terminated and the media were frozen until further P4 concentration analysis. GH (100 ng/ml) increased P4 secretion by CL1 and CL2 and had no effect on CL3. In separate studies these cells were treated for 48 h with IGF-I alone or with GH combined with IGF-I. IGF-I alone increased basal P4 secretion only by cells collected from CL1 while concurrent treatment with GH had no effect on P4 secretion by any type of CL. To investigate the possible mechanism of GH and IGF-I mediated induction of P4 secretion, an inhibitory study was conducted. In this experiment, luteal cells collected from CL1 were cultured in the absence or presence of cycloheximide (an inhibitor of protein synthesis) or actinomycin D (an inhibitor of DNA transcription). Cycloheximide or actinomycin D completely blocked the stimulatory effect of both GH and IGF-I on P4 production but did not reduce basal progesterone secretion suggesting involvement of gene transcription and translation in the GH and IGF-I action on luteal cells. Additionally, the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) under the influence of GH added alone or together with IGF was measured by the conversion of pregnenolone to progesterone. Stimulation of P4 secretion in P5-treated cells in GH-stimulated cultures was not observed, however, high stimulatory effect was noted in IGF-I treated cultures. In conclusion, the present studies indicate that there is direct and cycle stage dependent influence of GH and IGF-I on steroidogenesis in porcine luteal cells. It is suggested that both IGF and GH may exert some regulatory action during CL development in the pig.

Key words: Growth hormone, insulin-like growth factor-I, corpus luteum, progesterone secretion, pig

[*]Corresponding author; E-mail: greg@zuk.iż.uj.edu.pl; Fax: (4812) 6343716

Growth hormone (GH) is a pituitary hormone involved in steroidogenesis in the corpus luteum (CL). In most species ovarian follicles and corpora lutea are sites of GH action because GH receptor (GHR) is found within granulosa cells as well as in the CL. Bramley and Menzies (1987) showed that homogenates of pig CL contained specific, high-affinity receptors for human GH. Later, Yuan and Lucy (1996) detected mRNA for the GHR within mRNA isolated from the porcine luteal tissue. They showed higher amount of mRNA for GHR on day 10 of the oestrous cycle and decreasing amount of mRNA for GHR on day 19. GH might interact with its own receptors and directly accomplish its effect. Also, GH could stimulate ovarian production of IGF-I, which in turn mediates GH effects (Hsu and Hammond, 1987; Hynes et al., 1987).

Many of the effects of GH are mediated by insulin-like growth factor-I which is synthesised in the liver and ovary in response to GH. There is a specific difference in the reproductive effects of GH. Yuan and Lucy (1995) investigated the effects of GH and IGF-I on progesterone secretion by isolated small and large luteal cells collected during mid-pregnancy and showed that GH increased progesterone secretion by large porcine luteal cells. Moreover, they showed synergistic action of GH and IGF-I on progesterone secretion by this type of cells. Insulin-like growth factor-I is produced within the porcine CL and is thought to play an autocrine/paracrine role in CL development/function during the early luteal phase (Gadsby et al., 1996; Nicholson et al., 1999). Using human luteal cells from early and mid-luteal phase of the menstrual cycle, Apa et al. (1996) found that IFG-I was able to stimulate directly production of progesterone and additionally mediated the positive action of GH on progesterone synthesis. Measuring IGF-I production by the CL and then synergistic action with GH, FSH and hCG on progesterone secretion, Devoto et al. (1995) suggested that human CL is a site of secretion, action and reception of IGF-I during the mid-luteal phase.

Usually, the majority of data on steroid secretion by the CL concern particular days of the luteal phase. Such an approach does not provide the information about the dynamics of steroid secretion during the whole life span of the CL.

The presented study was conducted to investigate the interaction between GH and IGF-I on progesterone synthesis in cultured luteal cells. The mechanism of action of GH and IGF-I was also investigated.

Materials and methods

Chemicals

Parker medium (M199), calf serum, trypsin and PBS were obtained from Biomed, Lublin, Poland. Insulin-like growth factor (I 3769), pregnenolone (P 9129), antibiotic-antimycotic solution (A 9909) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant ovine growth hormone

(oGH) was prepared as in the Institute of Biochemistry, Food Science and Nutrition (Rehovot, Israel), described previously (Sakal et al., 1997).

Cell cultures

Ovaries obtained from Large White sows from a local slaughterhouse immediately after slaughter were placed in ice-cold PBS and transported to the laboratory. Dissected CL from each animal were enzymatically dissociated according to the technique of Gregoraszczuk (1983). Luteal cells were obtained from pools of freshly excised newly forming CL (ELP; 1–3 days after ovulation), mature corpora lutea (MLP; 10–14 days after ovulation) and regressing CL (LLP; 16–19 days after ovulation) from three animals. The phase of the oestrous cycle was determined according to the established morphological criteria (Gregoraszczuk, 1992). Classification was confirmed by histological comparison of representative CL from the abattoir with CL taken from pigs slaughtered at known intervals after observed oestrus.

Cells were suspended in medium M199 supplemented with 5% of calf serum at a concentration of 3.5×10^5 cells/ml medium. Cell viability measured using the trypan blue exclusion test was 85%. Cells were grown in multiwell plates (Nunc) in a humidified atmosphere with 5% CO_2 in the air. At least three different experiments (n = 3), each in triplicate, have been done.

Experimental procedure

To examine oestrous cycle dependent GH action on progesterone secretion, cells isolated from CL collected at three different periods of the luteal phase were plated separately into 24-well plates and cultured for 48 h in the control medium or incubated with different doses of GH (10, 100 or 200 ng/ml). After 48 h all cultures were terminated and the media were frozen until further progesterone analysis (Exp. 1; n = 5 pigs).

In separate studies (Exp. 2; n = 4 pigs), to examine GH amplification of IGF-I induced progesterone secretion, cells were cultured for 48 h with no hormones (control), or with 100 ng/ml of GH, 30 ng/ml of IGF-I, or GH combined with IGF-I at the above concentrations. After 48 h all cultures were terminated and the media were frozen until further progesterone analysis.

To investigate the possible mechanism of GH and IGF-I action on the induction of progesterone secretion by its influence on gene transcription and translation, an inhibitory study was conducted (Exp. 3; n = 4 pigs). In this experiment, luteal cells were cultured in the absence or presence of cycloheximide (an inhibitor of protein synthesis, 3 μg/ml for 24 h or actinomycin D (an inhibitor of DNA transcription, 1 μg/ml for 24 h) with or without GH. The doses of cycloheximide and actinomycin were chosen according to Xu et al. (1997).

The activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) under the influence of GH added alone, IGF-I added alone or GH combined with IGF-I in cells collected from early CL was measured by the conversion of pregnenolone (P5; 10 μg/ml) to progesterone (Exp. 4; n = 3 pigs). The net synthesis and secretion of progesterone to the culture medium was used as the indicator of 3β-HSD activity (Bar-Ami and Gitay-Goren, 2000; Miszkiel and Kotwica, 2001). Trilostane, the inhibitor of 3β-HSD in a dose of 100 μM, established on the basis of the dose-response curve performed during the preliminary studies (Gregoraszczuk et al., 1999; Gregoraszczuk et al., 2000), was used to confirm the effect. Trilostane has been shown to be the competitive inhibitor of 3β-HSD from several different sources (Naville et al., 1991; Cooke, 1996).

Progesterone concentration analysis

Progesterone concentration was determined radioimmunologically using Spectra kits (Orion Diagnostica, Finland), supplied by Polatom (Świerk, Poland). The limit of assay sensitivity was 94 pg/ml. The coefficients of variation between and within assays were 5.8% and 2.9%, respectively. The mean recoveries were 95.1–103.7%. The cross-reaction with pregnenolone was 2.9%. All other tested steroids (5β-dihydroprogesterone, 20β-hydroxyprogesterone, corticosterone, testosterone and oestrone) showed less than 1% cross-reaction.

Statistical analysis

All data points are expressed as means ± SEM from at least three different experiments (n = 3), each in triplicates. Significance of differences between the concentrations of progesterone in the control and experimental cultures were compared by analysis of variance and by using Duncan's new multiple range test.

Results

Oestrous cycle dependent GH action on progesterone secretion (Exp. 1)

GH in doses of 10 and 200 ng/ml had no effect on progesterone secretion by luteal cells collected from all investigated types of CL. In a dose of 100 ng/ml of GH a 1.6-fold increase (5.89 ng/ml vs. 3.7 ng/ml in the control culture) in basal progesterone secretion by cells collected from ELP, a 4.7-fold increase (4.31 ng/ml vs. 0.91 ng/ml in the control culture) in basal progesterone secretion by cells collected from MLP and no effect on its secretion by cells collected from LLP were noted (Fig. 1).

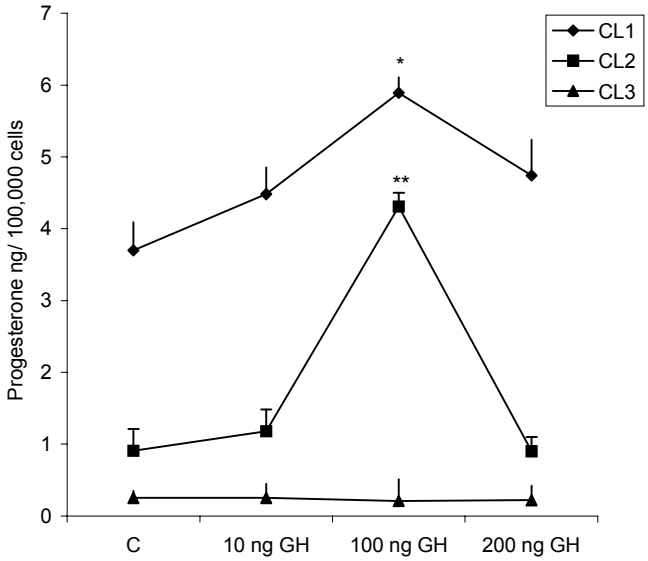

Fig. 1. Effect of graded concentrations (10, 100 and 200 ng/ml) of ovine growth hormone (oGH) on progesterone secretion by porcine luteal cells isolated from corpora lutea during the early (CL1), mid- (CL2) and late luteal phase (CL3) (Exp. 1). Treatments marked with an asterisk are different from their respective controls ($^{*}p < 0.05$; $^{**}p < 0.01$)

Effect of treatment with GH on IGF-I stimulated progesterone secretion (Exp. 2)

IGF-I increased basal progesterone secretion by cells collected from ELP (6.89 ng/ml vs. 3.74 ng/ml of the control) while it had no effect on progesterone secretion by cells collected from MLP and LLP (Fig. 2). Concurrent treatment with GH had no effect on progesterone secretion by any type of CL. Moreover, abolition of the stimulatory effect of both hormones on progesterone secretion by cells collected from ELP was noted (Fig. 2).

Effect of GH on progesterone secretion by its influence on gene transcription and translation (cycloheximide or actinomycin D inhibitory study; Exp. 3)

Cycloheximide or actinomycin D completely blocked the stimulatory effect of both GH and IGF-I on progesterone production but did not reduce basal progesterone secretion. The highest inhibitory effect was noted in GH + IGF-I stimulated cells (Fig. 3).

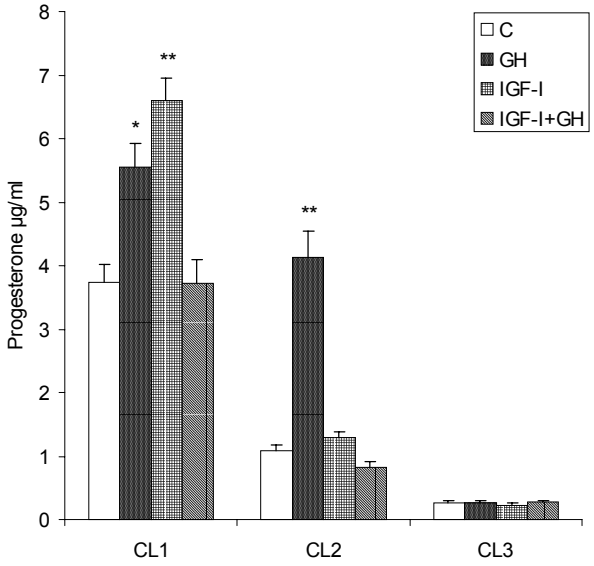

Fig. 2. The combined action of oGH (100 ng/ml) and IGF-I (30 ng/ml) on progesterone secretion by porcine luteal cells isolated from corpora lutea during the early (CL1), mid- (CL2) and late luteal phase (CL3) (Exp. 2). Treatments marked with and asterisk are different from their respective controls ($^{*}p < 0.05$; $^{**}p < 0.01$)

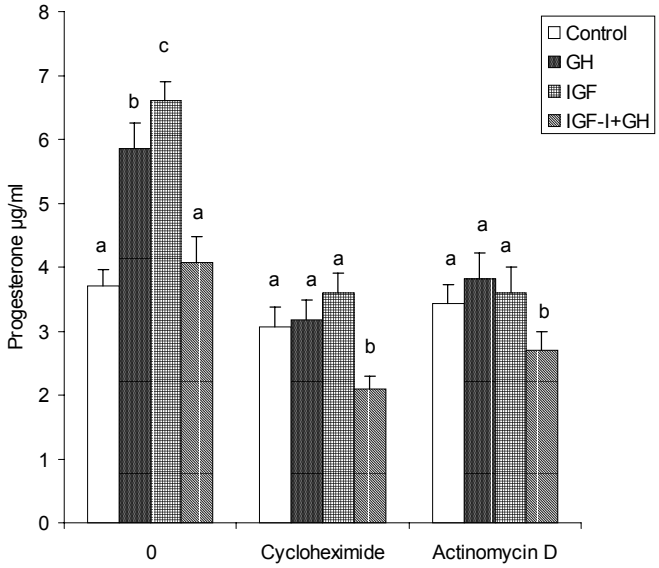

Fig. 3. Inhibitory effects of cycloheximide or actinomycin D on GH alone, IGF-I alone or combined treatment progesterone secretion (Exp. 3). Letters refer to comparison of progesterone secretion among treatments (a–a: $p < 0.05$; a–b: $p < 0.01$; a–c: $p < 0.001$)

GH and IGF-I action on Δ^5, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity (trilostane inhibitory study; Exp. 4)

Addition of the substrate for progesterone secretion to the luteal cells caused a 2-fold increase in progesterone secretion. No additional stimulation of progesterone secretion in pregnenolone- (P5-) treated cells in GH-stimulated cultures was observed. However, high stimulatory effect was noted in IGF-I treated cultures (13.2 μg/ml in P5 + IGF-I treated cells vs. 6.6 μg/ml in IGF-I treated cultures, and 3.7 μg/ml in the control culture). In GH-stimulated cells trilostane decreased progesterone secretion to 20% of the basal level while to 16% in P5-stimulated progesterone secretion. In IGF-I stimulated cells trilostane decreased progesterone secretion to 2% of the basal secretion and to 2.8% in P5-stimulated progesterone secretion (Fig. 4).

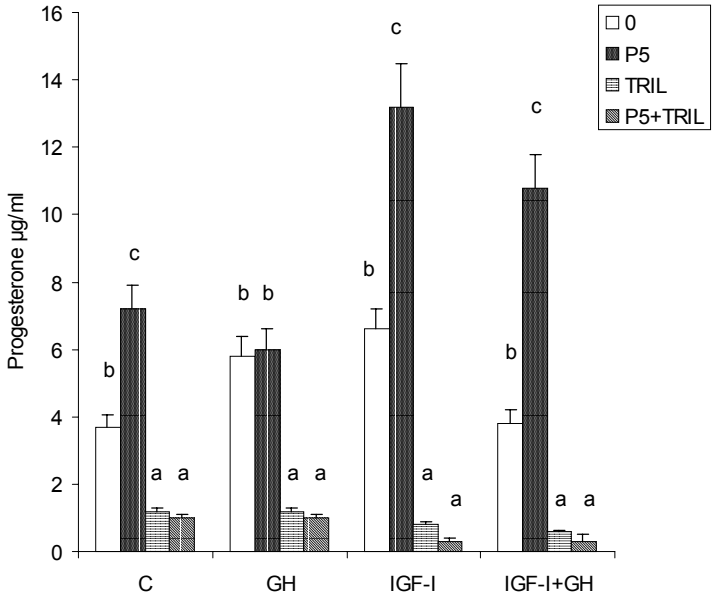

Fig. 4. The influence of the 3β-HSD inhibitor trilostane on GH (100 ng/ml) alone, IGF-I (30 ng/ml) alone or added in combination on basal (C) and pregnenolone- (P5-) stimulated progesterone secretion (Exp. 4). Letters refer to comparison of progesterone secretion among treatments (a–a: $p < 0.05$; a–b: $p < 0.01$; a–c: $p < 0.001$)

Discussion

In the early 1990s receptors for GH or mRNA encoding GH receptor were identified in bovine (Lucy et al., 1993), rat (Carlsson et al., 1993) and ovine (Juengel et al., 1997) luteal tissue. Although a number of physiological studies

have implicated GH in altering ovarian function in cows, rats and ewes, there are relatively few data concerning pigs (Hsu and Hammond, 1987; Yuan and Lucy, 1996; Xu et al., 1997; Gregoraszczuk et al., 2000).

The results of the presented study demonstrate that cultured luteal cells were responsive to GH during the early and middle luteal phase while they did not respond to GH during the late luteal phase. Small stimulatory effect of 10 ng/ml GH, higher stimulatory effect of 100 ng/ml and no effect of 200 ng/ml GH was noted. The occurrence of U-shape dose-response relationships (often termed hormesis) has been documented in numerous investigations (Calabrese and Baldwin, 2001). The luteal-phase-dependent action of GH observed in this study is in agreement with data of Yuan and Lucy (1996) who determined mRNA expression for GH receptor in porcine luteal tissue and showed higher amount of mRNA for GHR and 3β-HSD in the CL on day 10 of the oestrous cycle and a decrease on day 19. Unfortunately, they did not collect luteal cells from early developing CL.

GH may influence luteal function indirectly by increasing expression of IGF-I, which is synthesised by the CL of many species. Indeed, in the ewe, GH supports IGF expression in the luteal tissue (Juengel et al., 1997). Receptors for IGF-I have been demonstrated in luteal tissues of several species including the rat (Talavera and Menon, 1991; Parmer et al., 1991), cow (Sauerwien et al., 1992), human (Obasiolu et al., 1992) and ewe (Perks et al., 1995). It has been suggested that the effects of GH in the ovary involve an elevation in the levels of IGF-I in both the serum of humans (Homburg et al., 1990) and in the follicular fluid in pigs (Spicer et al., 1992).

In the present study, we observed the highest stimulatory effect of IGF-I on progesterone secretion by cells collected from early, developing CL, a smaller one on those from the mid-luteal phase, and no effect on LLP. This is in agreement with the results of Nicholson et al. (1999) who showed that IGF-I is produced within the porcine CL and suggested an autocrine/paracrine role in CL development/function during the early luteal phase. A stimulatory effect of IGF-I on luteal progesterone production *in vitro* was noted by Huang et al. (1992) in the pig. Our results are in agreement with data reported by Gadsby et al. (1996) who discovered that IGF-I mRNA expression in small luteal cells was significantly greater before day 12 (days 4–10) than after day 12 (day 12–16) and are in accordance with subsequent data of Schams et al. (1999) and Ge et al. (2000) who detected IGF-I receptor in the pig CL, especially during the early luteal phase.

Moreover, results of the study presented here suggest that both GH and IGF-I act as stimulatory factors; however, no synergistic action of these agents was observed when they were added together to the culture medium. A new observation is that the synergistic action of IGF-I and GH is rather inhibitory. This last finding contrasts with the results obtained by Yuan and Lucy (1995) who showed that combinations of GH and IGF-I increased progesterone secretion by

large luteal cells collected from the CL on day 44 of pregnancy. On the other hand, these authors noted that similar combinations of GH and IGF-I had no effect on small luteal cells. In our experiments both large and small luteal cells were present in the cell suspension, thus creating more physiological conditions similar to those existing in the CL *in vivo*.

The presented data suggested no influence of GH on the activity of 3β-HSD as measured by the conversion of pregnenolone to progesterone. The trilostane inhibitory study showed that in GH-stimulated cells trilostane decreased progesterone secretion in the same manner as under control conditions, while a 7.2-fold higher inhibition was noted in cultures supplemented with IGF-I . This is in accordance with data of Juengel et al. (1994, 1995) who showed that treatment of hypophysectomised ewes with GH alone also allows circulating concentrations of progesterone and expression of mRNA encoding StAR and cholesterol side chain cleavage P450 scc to reach normal levels, however, GH does not appear to support luteal expression of mRNA encoding 3β-HSD. On the other hand, in the presented data high stimulatory effect was noted in IGF-I treated cultures (Figs 3 and 4). De Moura et al. (1997) showed that IGF-I increased basal levels of 3β-HSH transcription in rat granulosa cells. However, our observation in porcine luteal cells is new and suggest that IGF-I enhances progesterone secretion by luteal cells through up-regulation of the activity of key enzyme in the steroidogenic pathway.

An inhibitory study showed that the presence of cycloheximide or actinomycin D completely blocked the stimulatory effect of both GH and IGF-I on progesterone production. However, it did not reduce basal progesterone accumulation, suggesting involvement of gene transcription and translation in the action of both GH and IGF-I in porcine luteal cells. This confirms earlier data of Xu et al. (1997) who suggested involvement of gene transcription and translation in IGF-II induced progesterone accumulation and amplification of GH on IGF-II induced progesterone secretion by porcine granulosa cells.

In conclusion, the present studies indicate that the action of GH and IGF-I on progesterone accumulation depends on the stage of the luteal phase. We suggest that the actions of GH and IGF-I on porcine luteal function in an early phase of its development are achieved directly through their own receptors and are not mediated by ovarian IGF-I synthesis. We propose that IGF-I induced progesterone accumulation by a stimulation of 3β-HSD activity while in the case of GH stimulation of progesterone accumulation was independent of the activation of this enzyme. The differences in the activation of P450 scc mRNA under the influence of GH and IGF-I in porcine granulosa cells were noted by Xu et al. (1997). They observed that porcine GH alone induced no stimulation of the P450 scc while IGF-II stimulated P450 scc mRNA levels. Finally, we suggested that both the IGF system, as was also suggested by Gadsby et al. (1996), and GH may have an autocrine/paracrine regulatory action on CL development and function in the pig.

Acknowledgements

We thank Dr. Maria Mika from the Department of Animal Physiology, Academy of Agriculture, Kraków for help in the RIA, Prof. Arie Gertler (Institute of Biochemistry, Food Science and Nutrition, Rehovot, Israel) for his generous gift of oGH and o IGF-I. This work was supported by the DS/IZ/2002 grant.

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